Evaluation of potential reproductive and developmental toxicity of potassium perfluorohexanesulfonate in Sprague Dawley rats

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ABSTRACT

This study evaluates the potential reproductive and developmental toxicity of perfluorohexanesulfonate (PFHxS), a surfactant found in sera of the general population. In a modified OECD 422 guideline-based design, 15 rats per sex and treatment group (control, 0.3, 1, 3, and 10 mg/kg-d) were dosed by gavage with potassium PFHxS (K^+PFHxS) or vehicle (0.5% carboxymethylcellulose) 14 days prior to cohabitation, during cohabitation, and until the day before sacrifice (21 days of lactation or presumed gestation day 25 (if not pregnant) for females and minimum of 42 days of treatment for males). Offspring were not dosed by gavage but were exposed by placental transfer in utero and potentially exposed via milk. Evaluations were made for reproductive success, clinical signs, body weight, food consumption, estrous cycling, neurobehavioral effects, gross and microscopic anatomy of selected organs, sperm, hematology, clinical pathology, and concentration of PFHxS in serum and liver. Additional three rats per sex per group were added to obtain sera and liver samples for PFHxS concentration determinations during the study. No reproductive or developmental effects were observed. There were no treatment-related effects in dams or offspring. K^+PFHxS-induced effects noted in parental males included: (1) at all doses, reductions in serum total cholesterol; (2) at 0.3, 3, and 10 mg/kg-d, decreased prothrombin time; (3) at 3 and 10 mg/kg-d, increased liver-to-body weight and liver-to-brain weight ratios, centrilobular hepatocellular hypertrophy, hyperplasia of thyroid follicular cells, and decreased hematocrit; (4) at 10 mg/kg-d, decreased triglycerides and increased albumin, BUN, ALP, Ca^{2+}, and A/G ratio. Serum and liver concentrations of PFHxS are reported for parents, fetuses, and pups. PFHxS was not a reproductive or developmental toxicant under study conditions.

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1. Introduction

Perfluorohexanesulfonate (PFHxS) is a perfluorinated surfactant in which all hydrogens in carbon–hydrogen bonds have been replaced with fluorine. As such, PFHxS is one of the number of functionalized, polyfluorinated compounds that have been produced for over half a century for use in specialized applications [1] as well as becoming the subject of increasing investigation with regard to environmental and health-related properties [2–5]. The unique properties of this and other perfluorinated surfactants, such as high surface activity, exceptional stability to degradation, density, solubility characteristics, and low intermolecular interactions, have been exploited in numerous industrial and consumer applications [6].

However, these same properties also create challenges for managing these materials in the environment. Exceptional stability

to environmental and metabolic degradation together with poor elimination from the body in the case of several perfluorinated surfactants [7–9], including PFHxS, creates a potential for accumulation and biomagnification. The geometric mean serum PFHxS elimination half-life in 26 retired production workers was estimated to be approximately 7.3 years (95% CI = 5.8–9.2) [7]. Spliethoff et al. [10] estimated the serum elimination half-life for PFHxS to be 8.2 years (95% CI = 5.4–16.2) using PFHxS concentration data from neonatal screening blood spots. Although the reported half-life values from the latter two articles are imprecise estimates, they are consistent in suggesting poor elimination of PFHxS in humans. Due to these properties, it may not be surprising that Hansen et al. [11] found PFHxS in pooled serum from the United States general population, and numerous later biomonitoring studies have found PFHxS widely distributed at low ng/mL concentrations in individual samples from the general population [10,12–26].

The presence of PFHxS in umbilical cord blood at birth [19] and neonatal screening program blood spots [10] demonstrates that human exposures can begin in utero. The primary sources of exposure are not understood; however, exposure to PFHxS in human

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milk [20–22], house dust [27,28] or from contact with surfaces treated with PFHxS-containing carpet and upholstery protection formulations [14] may provide some contribution as well as potential dietary [24] and water sources [23,26,29]. These sources may contribute to PFHxS exposure to children after birth.

Between the years 2000 and 2002, due to persistence and evidence of widespread exposure of the general population, 3M Company discontinued production of PFHxS along with perfluorooctanoate (PFOA) and chemistries based on perfluorooctanesulfonyl fluoride, including perfluorooctanesulfonate (PFOS). Several recent biomonitoring studies have demonstrated that blood concentrations of PFHxS, PFOA, and PFOS in the general population have declined significantly since 3M Company ceased manufacturing of these materials [10,13,17].

Although its eight-carbon analog, PFOS, has been extensively studied for its potential heath effects [2,30,31], little information on the toxicity potential of PFHxS has been documented. The fact that exposure occurs *in utero* and is present at all life stages together with the known biological effects profile of PFOS from laboratory studies warrants a need to understand potential health risk associated with exposure. The objective of this study was to evaluate the potential reproductive, developmental, and neurological responses of rats to treatment with potassium PFHxS in an assessment based on OECD Method 422 guidelines [32]. Because internal dose metrics have become important in evaluating health risk [2,33–35], collection of serum and liver PFHxS concentration data was included in the study design. One of the earliest clinically measurable indicators of biological response to treatment of rats and non-human primates with PFOS has been reduction in serum cholesterol [36,37]. Accordingly, serum total cholesterol was included in the clinical chemistry profile. We believe that this is the first article to report on the biological response to treatment with PFHxS.

2. Materials and methods

2.1. Test article (K⁺PFHxS), vehicle, and preparation of dosing solutions

K⁺PFHxS (purity 99.98%), a white powder, was obtained from 3M Company (St. Paul, MN). The vehicle used in preparation of the test article formulations and for administration to the control group was 0.5% carboxymethylcellulose, medium viscosity (lot number 120K0252, Sigma–Aldrich, St. Louis, MO) in deionized water. Dosing formulations for four K⁺PFHxS treatment levels of 0.3, 1, 3, and 10 mg/kg-d were prepared as K⁺PFHxS concentrations in vehicle of 0.03, 0.1, 0.3, and 1 mg/mL, respectively, based on a dosing volume of 10 mL/kg and were not adjusted for purity. The purity and stability of the chemical were verified by analysis before the study. All samples were analyzed for PFHxS concentration using a high performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) method.

2.2. Animal husbandry, assignment, and breeding

Crl:CD®(SD)IGS BR VAF/Plus® rats (n = 90/sex) supplied by Charles River Laboratories from St. Constant, Quebec, Canada (male rats) and Raleigh, NC, US (female rats) were used in this study. All rats were individually housed in stainless steel, wire-bottomed cages except during cohabitation and postpartum periods. During cohabitation, each pair of male and female rats was housed in the male rat's home cage. F₀ generation female rats were individually housed in nesting boxes (with Bed-o'cobs® bedding, The Andersons Industrial Products Group, Maumee, OH) until they either naturally delivered litters or were sacrificed. Each dam and her delivered litter were housed in a common nesting box during the postpartum period. All cage sizes and housing conditions were in compliance with the *Guide for the Care and Use of Laboratory Animals* [38].

Rats were given *ad libitum* access to Certified Rodent Diet #5002 (PMI Nutrition International, Inc., St. Louis, MO) except for the evening prior to sacrifice. Municipal water, processed through reverse osmosis membrane and fortified with chlorine, was given to the rats *ad libitum*. The study room was maintained under conditions of positive airflow with a minimum of ten changes per hour of air that passed through 99.97% HEPA filters. Room temperatures were maintained between 18 °C and 26 °C and relative humidity maintained at 30–70%. Lighting was maintained at 12-h light and 12-h dark cycles. The testing facility (Charles River Preclinical Services, Horsham, PA) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures involving animals were approved by the testing facility's Institutional Animal Care and Use Committee.

2.3. Study design

This study was conducted according to OECD test guideline 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test [32]). Male and female rats (n = 18/sex/dose) were allocated randomly into five oral gavage treatment groups consisting of the vehicle control, and 0.3, 1, 3, and 10 mg/kg-d K⁺PFHxS. In each treatment group, rats were assigned to either the main study (15 rats/sex) or for collection of sera and liver samples for PFHxS concentration determination (3 rats/sex). The dosage volume (10 mL/kg) was adjusted daily on the basis of the individual body weights recorded before intubation. Rats were dosed at approximately the same time each day. Daily dosing of all rats began 14 days prior to cohabitation and continued until the day prior to scheduled sacrifice. F₁ generation pups were not dosed directly but were exposed to PFHxS *in utero* and were potentially exposed via the milk during the lactation period.

Male rats were sacrificed on study day (SD) 42 (3 males assigned for PFHxS concentration determinations) and SD 44 (main-study males). For main-study female rats, scheduled sacrifice was either through postnatal day (PND) 22 or presumed gestation day (GD) 25 for rats that did not deliver a litter. Female rats assigned for collection of sera and liver samples for PFHxS concentration determination were sacrificed on GD 21. F₁ pups were sacrificed on PND 22. Carbon dioxide asphyxiation was used for sacrifice.

2.4. Observations and evaluations (F₀ parental rats)

All main study rats were observed daily for body weight (except during cohabitation) and clinical signs of toxicity (before and approximately 60 min after dosing), and detailed clinical observations were conducted on a weekly basis. Feed consumption was evaluated weekly for males except during cohabitation. Feed consumption for females was evaluated up to cohabitation and on GD 0, 7, 10, 12, 15, 18, and 20 and PND 1, 5, 8 and 15. During parturition, female rats were evaluated for adverse clinical signs, duration of gestation, litter sizes (all pups delivered), and pup viability at birth. Maternal behavior was evaluated on PND 1, 5, 8, 15, and 22.

Estrous cycling was evaluated in all female rats (including rats assigned to sera and liver sample collection) by examination of vaginal cytology after the first dose and daily until spermatozoa were observed in a vaginal smear and/or a copulatory plug was observed *in situ* during the cohabitation period.

In each treatment group, functional observation battery (FOB) and motor activity assessments were conducted on 10 male rats (prior to scheduled sacrifice and before blood sample collection) and 10 female rats (prior to scheduled sacrifice during lactation period) [39–42]. The FOB evaluations were conducted in a blinded manner with the following parameters assessed: lacrimation, salivation, palpebral closure, prominence of the eye, pupillary reaction to light, piloerection, respiration, and urination and defecation (autonomic functions); sensorimotor responses to visual, auditory, tactile and painful stimuli (reactivity and sensitivity); reactions to handling and behavior in the open field (excitability); gait pattern in the open field, severity of gait abnormalities, air righting reaction and landing foot splay (gait and sensorimotor coordination); forelimb and hindlimb grip strength; and abnormal clinical signs including convulsions, tremors and other unusual behavior, hypotonia or hypertonia, emaciation, dehydration, unkempt appearance and deposits around the eyes, nose or mouth. Motor activity was evaluated by a passive infrared sensor. Each test session was 1.5 h in duration with the number of movements and time spent in movement tabulated at each 5-min interval.

Gross necropsy of all male and female rats included macroscopic evaluation of external surfaces and all orifices, as well as cranial, thoracic and abdominal cavities, and their contents for gross lesions. The reproductive organs were closely examined (see below).

Ten rats per sex from each treatment group that were assigned to FOB and motor activity testing were used for evaluation of organ weights, microscopic histological examination of tissues, detailed examination or sexual organs and related parameters, clinical chemistry, and hematology. Procedural descriptions follow.

Organ weights, including sexual organ weights, were determined, and microscopic histological samples were taken for potential examination. Liver, kidneys, adrenals, thymus, testes (left and right), epididymis (left and right), seminal vesicles (with and without fluid), prostate, spleen, brain, heart, ovaries (left and right) and uterus were weighed. Brain, small and large intestines, lungs, lymph nodes, peripheral nerve, stomach, kidneys, spleen, thymus, trachea, urinary bladder, spinal cord, liver, adrenals, heart, thyroid/parathyroid, bone marrow, testes, prostate, seminal vesicles, the remaining portion of the left epididymis, the right epididymis, ovaries, uterus, vagina, mammary gland and any gross lesions were retained in neutral buffered 10% formalin and evaluated histologically by Research Pathology Services, Inc. (New Britain, PA). The control and 10 mg/kg-d treatment groups were evaluated first. If differences were found between control and the high treatment group, the remaining three treatment groups were to be evaluated.

In addition to organ weights, potential toxicity to male and female reproductive systems was closely evaluated. For male rats, sperm concentration (per gram of tissue weight), sperm motility (by integrated visual optical system, or IVOS, Hamilton Thorne, Inc., Beverly, MA), and sperm morphology (by determining the percentage of normal and abnormal sperms) were assessed. Uteri of both pregnant and non-pregnant rats were examined for the presence/absence of and the number (if

present) of implantation sites. A quantitative evaluation of primordial follicles was also conducted for F_0 generation female rats.

Hematology and clinical chemistry samples were collected from the inferior vena cava of fasted rats assigned to the FOB assessment in the main study during the scheduled sacrifice. All samples were analyzed by Redfield Laboratories, Redfield, AK (a division of Charles River Laboratories).

Analyses of hematologic parameters included erythrocyte count (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), total leukocyte count (WBC), and differential leukocyte count, platelet count (PLAT), mean platelet volume (MPV), cell morphology, prothrombin time (PT), and activated partial thromboplastin time (APTT).

The evaluated clinical chemistry parameters were: total protein (TP), triglycerides (TRI), albumin (A), globulin (G), albumin/globulin ratio (A/G), glucose (GLU), cholesterol (CHOL), total bilirubin (TBILI), urea nitrogen (BUN), creatinine (CREAT), creatinine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK), calcium (CA), phosphorus (PHOS), sodium (NA), potassium (K), and chloride (CL).

Blood samples (processed to plasma) were collected from three male rats per treatment group on SD 14 and 42. Blood samples were also collected from the three female rats per treatment group on SD 14 and GD 21. Samples were collected from the orbital sinus on SD 14 and from the vena cava on SD 42. After the last blood collection on SD 42, all three male rats were euthanized and liver samples were harvested and weighed. Liver samples were also collected and weighed from the three female rats euthanized on GD 21 followed by the subsequent collection of fetal blood and liver (pooled by litter) samples. All samples were stored frozen until PFHxS concentration analysis (Exygen Research, State College, PA).

2.5. Observations and Evaluations (F_1 Generation)

The F_1 pups in each litter were counted once daily and clinical observations were recorded daily. Pup body weights were recorded on PND 1, 8, 15 and 22. No feed consumption or interim body-weight data were recorded prior to the scheduled sacrifice on PND 22. Pups were examined for gross lesions at sacrifice, and the necropsy also included a single cross-section of the head at the level of the frontal-parietal structure and examination of the cross-sectioned brain for apparent hydrocephaly.

At study termination, liver samples were excised and weighed and blood samples were collected and processed for serum from five pups per sex per litter from the 10 dams selected for FOB assessments. Serum samples were pooled by litter. The median liver lobe was stored frozen, and the remaining liver portion was retained in neutral buffered 10% formalin for possible histological evaluation. Samples remained frozen until PFHxS concentration analysis (Exygen Research, State College, PA).

2.6. Determination of PFHxS Concentrations in Serum and Liver

PFHxS concentrations were determined by LC–MS/MS. All samples underwent extraction procedures prior to chromatographic analyses. Blank serum and liver samples were purchased from Pel Freeze Biologicals (Rogers, AR) and these blank matrices were used to prepare the appropriate matrix-matched PFHxS standard curves with the addition of known amounts of PFHxS.

Serum (100 µL) and liver (100 mg) samples were diluted with deionized water so that the final volumes were 20 mL and 10 mL, respectively. After vortexing (serum and plasma) and homogenization (liver), 1 mL of the aliquot was mixed with 5 mL acetonitrile using a mechanical shaker (20 min, room temperature) and supernatant was obtained after centrifugation (3000 rpm, 5 min). After adding 35 mL of deionized water to the supernatant, the entire mixture was loaded onto Waters Sep-Pak® solid phase extraction (SPE) column (6 mL with 1 g C18 sorbent). The SPE columns were pre-conditioned with 10 mL methanol followed by 5 mL deionized water. Samples were eluted with 2 mL methanol.

The instrument used for PFHxS analysis was a Micromass Quattro Ultima mass spectrometer configured with electrospray ionization source (ESI) in negative ion mode. A Genesis® C8, 4 µm, 50 mm × 2.1 mm i.d. HPLC column with a flow rate of 0.3 mL/min was used for PFHxS analysis. The gradient condition for the mobile phase started with 90% 2 mM ammonium acetate and 10% methanol to 100% methanol for 9.5 min. All source parameters were optimized under these conditions according to manufacturer's guidelines. Transition ions monitored for PFHxS were from $399 \rightarrow 80$ atomic mass units.

2.7. Statistical analysis

F_0 parental rat data were evaluated with the individual rat as the unit measured. Litter values were used in evaluation of pup data, as appropriate. Variables with interval or ratio scales of measurement, such as body weights, feed consumption values, latency and errors per trial scores in behavioral tests, and percent mortality per litter were analyzed as parametric data. Bartlett's Test of Homogeneity of Variances [43] was used to estimate the probability that the dosage groups have different variances. A non-significant result ($p > 0.001$) indicated that an assumption of homogeneity of variance was appropriate, and the data were compared using the Analysis of Variance (ANOVA) [44]. If ANOVA test was significant ($p \leq 0.05$), the groups given

the test substance were compared with the control group using Dunnett's Test [45]. If Bartlett's Test was significant ($p \leq 0.001$), the ANOVA was not appropriate, and the data were analyzed as nonparametric data. When 75% or fewer of the scores in all the groups were tied, the Kruskal–Wallis Test [46] was used to analyze the data, and in the event of a significant result ($p \leq 0.05$), Dunn's Test [47] was used to compare the groups given the test substance with the control group. When more than 75% of the scores in any dosage group were tied, Fisher's Exact Test [48] was used to compare the proportion of ties in the groups.

Data from the motor activity test, with measurements recorded at intervals (blocks) throughout each test session, were analyzed using an ANOVA with repeated measures [49]. A significant result ($p \leq 0.05$) in that test could have appeared as effect of concentration (differences among dosage groups in the totals of all measurements in a session) or as an interaction between concentration and block (differences in the patterns of dosage group values across the measurement periods). If the concentration effect was significant, the totals for the control group and the groups given the test substance were compared using Dunnett's Test [45]. If the concentration × block interaction was significant, an ANOVA [44] was used to evaluate the data at each measurement period, and a significant result ($p \leq 0.05$) was followed by a comparison of the dosage groups using Dunnett's Test [45].

3. Results

All data reported in this study are presented as mean ± standard deviation (SD) unless specified otherwise.

3.1. Stability and homogeneity

Dosing solutions were found to be stable and homogenous. Mean PFHxS (and inferred K^+PFHxS) concentrations for seven sets of dosing solutions were within 70–125% of the target concentrations. The wide range of PFHxS concentrations obtained was likely due to the unavailability of appropriate internal standard at the time during which the analysis was conducted. (While the LC–MS/MS analytical coefficient of variation for PFHxS analysis typically ranges from 20 to 30% without employing an internal standard, it has been reduced significantly to less than 10% when an internal standard such as stable isotope ^{18}O-labeled PFHxS is used.)

3.2. F_0 parental rat results

All F_0 rats survived to the scheduled necropsy and no K^+PFHxS-treatment related mortalities occurred. No abnormal clinical findings were noted in any of the rats receiving either control or K^+PFHxS doses at the daily examinations or 1 h following dose administration.

Body weight and body-weight change data for F_0 males are summarized in Table 1. In males given K^+PFHxS, mean body weights by K^+PFHxS treatment level were not statistically significantly different than male control body weights at any time during the study. Statistically significant decreases in mean body-weight gains relative to mean control body-weight gains were noted in 0.3, 3, and 10 mg/kg-d K^+PFHxS-treated male groups between SD 29 (first value recorded after cohabitation) and 36, resulting in an overall decrease in body-weight gains through termination on SD 43, even though no differences from mean control values were noted between SD 36 and 43. Mean body-weight gain in the 10 mg/kg-d treatment group was decreased over the entire study period (SD 1–43) with statistical significance as compared to the mean for controls. Regardless of these variations in body-weight gain, mean terminal body weights were not statistically significantly different between K^+PFHxS-treated males and controls.

Body weight and body-weight change data for F_0 females also are summarized in Table 1. During pre-cohabitation and gestation, no statistically significant differences were noted in mean body weights or body-weight changes in K^+PFHxS-treated female groups when compared to the mean for controls for the same time periods. During lactation, there were no statistically significant differences between mean body-weight changes in K^+PFHxS-treated

Table 1

Body weights and body-weight changes summary for male and female parental rats during specified study periods (data in grams, N = 15 unless otherwise noted).

	Potassium perfluorohexanesulfonate dose (mg/kg-day)				
	0 (Control)	0.3	1	3	10
Male body-weight study day (SD) 1	342.7 ± 12.6	338.9 ± 13.1	341.9 ± 14.7	345.6 ± 11.2	343.3 ± 12.4
Male body-weight SD 43[a]	490.5 ± 37.2	475.1 ± 38.3	482.5 ± 53.1	490.5 ± 40.2	462.1 ± 22.0
Male body-weight SD 44[b]	472.5 ± 33.9	453.7 ± 34.3	463.1 ± 48.2	468.3 ± 37.1	441.5 ± 16.5
Male body-weight gain SDs 1–44[b,c]	129.8 ± 25.5	114.8 ± 27.5	121.2 ± 37.7	122.7 ± 30.0	98.2 ± 13.9**
Female body-weight SD 1	228.6 ± 8.0	224.7 ± 8.1	226.3 ± 7.7	226.8 ± 7.9	226.9 ± 8.0
Female body-weight SD 14	258.5 ± 16.1	250.7 ± 12.6	257.9 ± 13.6	253.0 ± 13.2	251.1 ± 18.0
Female body-weight gain SD 1–14[d]	29.9 ± 13.7	25.9 ± 7.2	31.6 ± 8.4	26.2 ± 8.3	24.2 ± 14.4
Maternal body-weight gestation day (GD) 0	270.1 ± 14.3	259.4 ± 12.0	270.9 ± 15.6[e]	265.6 ± 15.4[e]	260.9 ± 18.2
Maternal body-weight GD 20	412.0 ± 35.0	397.4 ± 27.8	412.1 ± 21.7[e]	398.8 ± 27.6[e]	395.1 ± 28.1
Maternal body-weight gain GD 0–20[d]	141.9 ± 29.0	138.0 ± 27.4	141.2 ± 14.1[e]	133.2 ± 21.9[e]	134.2 ± 16.9
Maternal body-weight postnatal day (PND) 1	313.1 ± 22.6	302.2 ± 16.8	309.5 ± 20.6[e]	304.7 ± 23.2[e]	296.3 ± 19.9
Maternal body-weight PND 8	342.3 ± 19.8	320.7 ± 12.6*	333.6 ± 19.6[e,f]	322.2 ± 21.5*	318.1 ± 26.0**
Maternal body-weight PND 15	355.3 ± 31.2	342.4 ± 10.4[g]	343.2 ± 17.2[e,f]	348.7 ± 21.4[e,f]	333.4 ± 33.6[g]
Maternal body-weight PND 21[a]	350.1 ± 26.9[g]	335.7 ± 18.7[g]	343.4 ± 19.8[e]	344.4 ± 25.8[e]	334.4 ± 35.9
Maternal body-weight PND 22[b]	306.3 ± 26.0	324.3 ± 27.1	311.2 ± 35.0[e]	319.6 ± 40.4[e]	303.5 ± 37.9
Maternal body-weight change (+/−) PND 1–22[h]	−6.9 ± 28.4	+22.1 ± 26.0	+1.7 ± 28.0[e]	+14.9 ± 31.8[e]	+7.3 ± 29.3

[a] Non-fasted body weight on last day before termination.

[b] Fasted terminal body weight.

[c] In males given potassium perfluorohexanesulfonate (K+PFHxS), mean body weights by treatment level were not statistically significantly different than male control body weights at any time during the study. Statistically significant decreases in mean body-weight gains relative to mean control body-weight gains were noted in 0.3, 3, and 10 mg/kg-d K+PFHxS-treated male groups between SD 29 (first value recorded after cohabitation) and 36 and from SD 29 through termination on SD 43; however, no differences from mean control values were noted between study days 36 and 43.

[d] During pre-cohabitation and gestation, no statistically significant differences were noted in mean body weights or body-weight changes in K+PFHxS-treated female groups when compared to the mean for controls for the same time periods.

[e] N = 13 due to 2 nonpregnant females.

[f] N = 12 due to exclusion of 2 body-weight determinations that appeared to be incorrect as a result of interrupted water access.

[g] N = 14 due to exclusion of 1 body-weight determination that appeared to be incorrect as a result of interrupted water access.

[h] During lactation, there were no statistically significant differences between mean body-weight changes in K+PFHxS-treated groups when compared to mean control values over the same time period. There were occasional periods of statistically significant differences in mean body weights among 0.3, 3, and 10 mg/kg-d K+PFHxS-treated groups and controls from PND 4–14. Statistically significant reductions in mean body weight occurred in K+PFHxS-treated females as compared to control mean values from PND 4 to PND 14 as follows: PND 4, 6–8, 11, and 13 in the 0.3 mg/kg-d group; PND 7 and 8 in the 3 mg/kg-d; and PND 4, 6–9, 11, 13, and 14 in the 10 mg/kg-d group. Mean body weights on PND 15 through term on PND 22 in K+PFHxS-treated groups were not statistically significantly different than control means.

* Statistically significantly different from control value ($p \leq 0.05$).

** Statistically significantly different from control value ($p \leq 0.01$).

groups when compared to mean control values over the same time period. There were occasional periods of statistically significant differences in mean body weights among 0.3, 3, and 10 mg/kg-d K+PFHxS-treated groups and controls from PND 4–14. Statistically significant reductions in mean body weight occurred in K+PFHxS-treated females as compared to control mean values from PND 4 to PND 14 as follows: PND 4, 6–8, 11, and 13 in the 0.3 mg/kg-d group; PND 7 and 8 in the 3 mg/kg-d; and PND 4, 6–9, 11, 13, and 14 in the 10 mg/kg-d group. Mean body weights on PND 15 through term on PND 22 in K+PFHxS-treated groups were not statistically significantly different than control means.

Treatment of male and female rats with K+PFHxS at doses up to 10 mg/kg-d did not affect mean absolute and relative feed consumption when compared to controls.

Mating and fertility data are presented in Table 2. K+PFHxS treatment did not affect any mating or fertility parameters investigated. Estrous cycling and all mating and fertility indices, including days in cohabitation, were unaffected by the K+PFHxS treatments up to 10 mg/kg-d.

Pregnancy status, gestation length, and pregnancy outcome are summarized in Table 3. Pregnancy occurred in 88.7–100% of rats in all treatment groups and all pregnant F_0 dams delivered lit-

Table 2

Mating and fertility values (mean ± SD) of F_0 male and female rats.

	K+PFHxS dose (mg/kg-d)				
	0	0.3	1.0	3.0	10.0
Male					
Number of animals	15	15	15	15	15
Cohabitation length (days)	3.2 ± 1.4	1.9 ± 1.2	2.8 ± 1.2	3.1 ± 2.0	2.5 ± 1.4
Mating index[a] (%)	14/15 (93.3%)	15/15 (100%)	13/15 (86.7%)	12/15 (80.0%)	15/15 (100%)
Fertility index[b] (%)	14/14 (100%)	15/15 (100%)	13/15 (86.7%)	12/13 (92.3%)	15/15 (100%)
Female					
Number of animals	15	15	15	15	15
Cohabitation length (days)	3.7 ± 3.0	1.9 ± 1.2	2.8 ± 1.2	4.0 ± 4.0	2.5 ± 1.4
Estrous cycle per 13-day	2.7 ± 0.9	2.8 ± 0.9	3.3 ± 0.6	2.9 ± 0.8	2.1 ± 1.2
Mating index (%)	15/15 (100%)	15/15 (100%)	13/15 (86.7%)	13/15 (86.7%)	15/15 (100%)
Fertility index (%)	15/15 (100%)	15/15 (100%)	13/15 (86.7%)	13/14 (92.8%)	15/15 (100%)

[a] Rats pregnant/number of rats in cohabitation.

[b] Pregnancies/number of rats that mated.

Table 3
Pregnancy outcome in F_0 female rats.

	K+PFHxS dose (mg/kg-d)				
	0	0.3	1.0	3.0	10.0
Number of rats assigned for mating	15	15	15	15	15
Number of rats successfully mated	15	15	15	14	15
Number of rats became pregnant	15	15	13	13	15
Pregnancy rates[a] (%)	100	100	86.7	92.9	100
Duration of gestation (days)	22.5 ± 0.5	22.7 ± 0.4	22.7 ± 0.5	22.8 ± 0.4	22.6 ± 0.5
Total implants	251	230	206	192	231
Litters delivered	15	15	13	13	15
Implants/litter	16.7 ± 1.3	15.3 ± 2.9	15.8 ± 1.3	14.8 ± 2.8	15.4 ± 2.3
Gestation index (%)	15/15 (100%)	15/15 (100%)	13/13 (100%)	13/13 (100%)	15/15 (100%)
Total number of pups delivered	235	214	201	181	220
Liveborn	235	214	200	177	218
Stillborn	0	0	1	3	2
Unknown vital status	0	0	0	1	0

[a] Pregnancy rate = number of rats became pregnant relative to the total number assigned (for mating).

ters. Pregnancy status and outcomes were unaffected by K+PFHxS treatments up to 10 mg/kg-d, the highest dose used in the study.

No test substance-related internal findings were observed for F_0 males and females at scheduled necropsies at any dose level. Macroscopic findings observed in the test article-treated groups occurred infrequently, at similar frequencies in the control group and/or in a manner that was not dose-related.

With the exception of liver weights in male rats, K+PFHxS treatment did not affect the mean absolute or relative weights of organs when compared to control. K+PFHxS treatment caused a statistically significant ($p < 0.05$) dose-related increase in mean absolute liver weight in male rats at doses of 3.0 and 10 mg/kg-d, which resulted in increases in mean liver weight of 20 and 56% over control, respectively. As a result, mean liver weight as a percent of body weight was also increased with statistical significance in male rats in the 3.0 and 10 mg/kg-d treatment groups (Fig. 1) as well as liver weight to brain weight ratio (data not shown). For female rats, K+PFHxS did not result in treatment-related changes in absolute or relative liver weights.

With the exception of liver and thyroid tissues in male rats, there were no differences in tissue histology between control and K+PFHxS-treatment groups. There were increased incidences of minimal to moderate hypertrophy seen in the liver and thyroid gland of male rats receiving 3.0 and 10 mg/kg-d K+PFHxS doses (Table 4). The affected centrilobular hepatocytes were enlarged with an increased amount of dense eosinophilic gran-

ular cytoplasm. The changes in the thyroid gland consisted of hypertrophy and hyperplasia of the follicular epithelium cells. These microscopic changes in the liver and thyroid were consistent with the known effects of compounds that cause microsomal enzyme induction where the hepatocellular hypertrophy results in a compensatory hypertrophy and hyperplasia of the thyroid due to increased plasma turnover of thyroxine and associated stimulation of thyroid-stimulating hormone in rats [50]. No treatment-related microscopic changes were observed in any of the female rats or male rats administered 0.3 or 1.0 mg/kg-d K+PFHxS doses.

Sperm motility, count, density, and morphology values for all F_0 male rats and primordial follicle counts for F_0 female rats (from control and 10 mg/kg-d K+PFHxS treatment group only) were unaffected by treatment with K+PFHxS.

Compared to controls, K+PFHxS treatments did not affect any of the hematology or clinical chemistry in F_0 female rats.

For F_0 male rats, the hematology and clinical chemistry parameters that were statistically different than the values reported for controls are summarized in Table 5. While the hematocrit and RBC counts were significantly decreased in the 3 and 10 mg/kg-d treatment groups, hemoglobin concentrations were significantly decreased in the 1, 3 and 10 mg/kg-d groups. Prothrombin time was significantly increased in the 0.3, 3 and 10 mg/kg-d dosage groups but not the 1.0 mg/kg-d group. While albumin, albumin/globulin ratio, blood urea nitrogen, alkaline phosphatase, and calcium were significantly increased in the 10 mg/kg-d treatment group, cholesterol values were significantly decreased in all treatment groups and triglycerides was significantly decreased in the 10 mg/kg-d treatment group only.
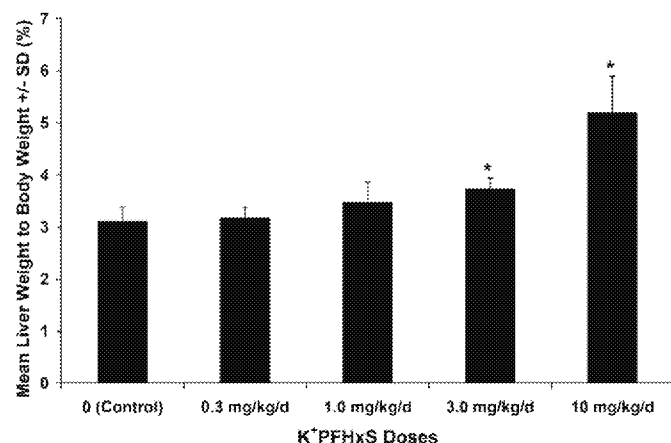

Fig. 1. Mean liver weight to body-weight percent data for F_0 male rats. Rats receiving daily K+PFHxS treatments at 3.0 mg/kg-d and 10.0 mg/kg-d had statistically significant increased liver weight to body-weight percent than controls. Asterisk (*) denotes significant difference from control ($p < 0.05$).

Table 4
Histopathology of the F_0 generation male rats.

	K+PFHxS dose (mg/kg-d)				
	0	0.3	1.0	3.0	10.0
Number of rats evaluated	10	10	10	10	10
Liver hypertrophy (hepatocellular, centrilobular)					
Minimal	0	0	0	8	4
Mild	0	0	0	1	5
Moderate	0	0	0	0	1
Total incidence	0	0	0	9	10
Thyroid hypertrophy/hyperplasia (follicular epithelium)					
Minimal	0	1	1	2	0
Mild	2	2	1	2	3
Moderate	0	0	0	0	4
Total incidence	2	3	2	4	7

Table 5
Hematological and clinical chemistry observations in male rats treated with K^+ PFHxS for at least 42 days.

	K^+ PFHxS dose (mg/kg-d)				
	0	0.3	1.0	3.0	10.0
Number of rats	10	10	10	10	10
Hematology					
Red blood cells ($10^6/mm^3$)	7.51 ± 0.40	7.33 ± 0.40	7.32 ± 0.44	$6.93 \pm 3.05^*$	$6.99 \pm 0.44^*$
Hemoglobin (g/dL)	16.5 ± 1.2	15.9 ± 0.4	$15.7 \pm 0.6^*$	$15.4 \pm 0.7^{**}$	$15.6 \pm 0.8^*$
Hematocrit (%)	43.5 ± 3.5	42.2 ± 1.6	42.0 ± 2.2	$40.2 \pm 2.3^{**}$	$40.7 \pm 1.8^*$
Prothrombin time (s)	13.4 ± 0.2	$14.2 \pm 0.3^{**}$	13.6 ± 0.2	$13.8 \pm 0.4^*$	$14.0 \pm 0.5^{**}$
Clinical chemistry					
Albumin (g/dL)	4.3 ± 0.2	4.1 ± 0.2	4.3 ± 0.2	4.2 ± 0.2	$4.5 \pm 0.2^{**}$
Albumin/globulin	2.1 ± 0.2	2.1 ± 0.2	2.2 ± 0.3	2.2 ± 0.2	$2.5 \pm 0.2^{**}$
BUN (mg/dL)	16.0 ± 1.5	16.0 ± 0.8	16.0 ± 1.8	17.0 ± 1.6	$21.0 \pm 2.4^{**}$
Creatinine (mg/dL)	0.30 ± 0.04	0.30 ± 0.05	0.30 ± 0.06	0.30 ± 0.03	0.30 ± 0.05
Calcium (mg/dL)	10.9 ± 0.5	10.7 ± 0.4	11.1 ± 0.4	11.1 ± 0.3	$11.5 \pm 0.4^{**}$
Sodium (mmol/L)	146.0 ± 1.4	146.0 ± 1.3	147.0 ± 1.6	147.0 ± 1.0	146.0 ± 2.2
Potassium (mmol/L)	6.6 ± 2.1	6.1 ± 0.9	6.5 ± 1.3	6.0 ± 0.6	6.9 ± 1.6
Chloride (mmol/L)	98.0 ± 2.7	99.0 ± 1.5	100.0 ± 2.3	100.0 ± 2.7	100.0 ± 1.2
Cholesterol (mg/dL)	57 ± 8	$41 \pm 11^{**}$	$46 \pm 12^*$	$43 \pm 13^{**}$	$33 \pm 7^{**}$
Triglycerides (mg/dL)	52 ± 21	47 ± 17	36 ± 14	36 ± 28	$17 \pm 8^{**}$
Alkaline phosphatase (U/L)	105 ± 14	111 ± 37	100 ± 12	115 ± 25	$144 \pm 38^{**}$
ALT (U/L)	42 ± 6	63 ± 70	60 ± 34	95 ± 124	45 ± 7
AST (U/L)	96 ± 22	121 ± 93	117 ± 41	198 ± 249	97 ± 16

[*] Significantly different from the control group value ($p \le 0.05$).
[**] Significantly different from the control group value ($p \le 0.01$).

There were no statistically significant differences between control and K^+ PFHxS treatments on the assessments of FOB parameters (autonomic functions, sensorimotor functions, excitability, gait and sensorimotor coordination and forelimb and hindlimb grip strength and abnormal clinical observations) and motor activity (data not shown).

3.3. F_1 Litter Results

In all K^+ PFHxS-treated groups, there were no statistically significant differences in litter outcomes when compared to the control values (given in parentheses) for the numbers of F_1 pups delivered (235 per 15 litters), liveborn pups (235), stillborn pups (0),

live pups on PND 1 (234), 8 (228), and 22 (228), and pups per litter on PND 22 (15.2 ± 1.8). The percent of pups that were male (45.2 ± 13.0), pup body weights (38.17 ± 4.72 and 36.67 ± 4.44 g for males and females, respectively), pup liver weights (1.48 ± 0.26 and 1.59 ± 0.24 g for males and females, respectively), liver-to-body-weight ratios (3.86 ± 0.27 and 4.33 ± 0.24 for males and females, respectively) on PND 22 also did not differ with statistical significance between control pups and those from K^+ PFHxS-treated groups. The viability indices (number of live pups on PND 8 relative to number of live pups on PND 1 as a percent) and lactational indices (number of live pups on PND 22 relative to number of live pups on PND 8 as a percent) were not statistically significantly different between pups from K^+ PFHxS-

Table 6
Mean serum/plasma and liver PFHxS concentration $\pm$ SD (ug/mL or ug/g) data summary.

		K^+ PFHxS dose (mg/kg-d)				
		0	0.3	1.0	3.0	10.0
Serum PFHxS concentrations (ug/mL)						
SD 14	F_0 male[a]	0.14 ± 0.05	18.18 ± 2.42	80.97 ± 30.83	143.05 ± 82.09	182.67 ± 8.25
	F_0 female[b]	0.39^c	2.78 ± 0.81	9.85 ± 3.91	20.67 ± 3.91	42.02 ± 6.47
SD 42	F_0 male	0.32 ± 0.09	44.22 ± 12.66	89.12 ± 0.80	128.67 ± 10.30	201.50 ± 20.02
GD 21	F_0 female	<LLOQ[d]	3.32 ± 0.71	10.65 ± 6.41	32.75 ± 7.83	59.80 ± 11.54
	F_1, pooled[e]	<LLOQ	5.32 ± 1.32	13.47 ± 2.06	37.10 ± 2.89	44.33 ± 6.50
PND 22	F_1, pooled	<LLOQ	8.57 ± 2.41	34.34 ± 10.86	32.35 ± 8.20	93.55 ± 55.79
Liver PFHxS concentrations (ug/g)						
SD 42	F_0 male	0.35 ± 0.23	43.80 ± 8.07	149.50 ± 26.06	338.67 ± 128.42	593.50 ± 81.41
GD 21	F_0 female	<LLOQ[f]	0.79 ± 0.19	2.61 ± 0.54	7.80 ± 1.58	16.53 ± 2.57
	F_1 fetus[g]	<LLOQ	1.37 ± 0.53	3.29 ± 1.17	7.19 ± 1.39	18.87 ± 4.28
PND 22	F_1 male[h]	<LLOQ	1.13 ± 0.31	3.86 ± 0.94	8.73 ± 1.65	16.22 ± 4.41
	F_1 female[i]	<LLOQ	1.04 ± 0.24	3.91 ± 1.05	9.96 ± 2.69	18.39 ± 2.32

[a] Parental male rats.
[b] Parental female rats (dams).
[c] Excludes data for two females that had serum measured below lower limit of quantification (LLOQ) of 0.1 μg/mL.
[d] Serum LLOQ = 0.1 μg/mL.
[e] GD 21 fetuses pooled by litter.
[f] Liver LLOQ = 0.1 μg/g.
[g] Individual GD 21 fetuses.
[h] Male pups.
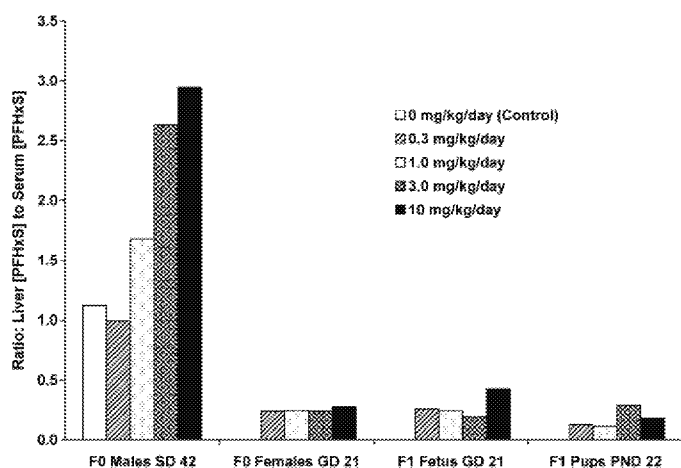[i] Female pups.

Fig. 2. Mean liver to serum PFHxS concentration ratio for F_0 males on study day (SD) 42, F_0 females on gestation day (GD) 21, F_1 fetus on GD 21, and F_1 pups on postnatal day 22. The F_1 generation rats did not directly receive K^+PFHxS treatment; therefore, presence of PFHxS in serum and liver was the result of *in utero* and lactational exposure.

treated and control groups (viability index = 97.4% and lactation index = 100%).

3.4. PFHxS concentrations in serum and liver

Mean PFHxS concentrations (±SD) in serum (or plasma) and livers are summarized in Table 6. The mean ratio between liver and serum PFHxS concentrations for each treatment groups are illustrated as Fig. 2.

4. Discussion

The purpose of this study was to provide information on the possible health hazards that may result from repeated exposure of Crl:CD®(SD)IGS BR VAF/Plus® male and female rats to K^+PFHxS beginning 14 days before cohabitation, through mating and continuing for at least 42 days (male rats) or through parturition until PND 21 (female rats). This repeated dose study incorporated a reproduction/developmental toxicity test that provided initial information on possible effects on male and female reproductive performance (e.g., gonadal function, mating behavior, conception, development of the conceptus and parturition). The study also included an evaluation of potential neurological effects in parental rats.

The overall body-weight gain from 10 mg/kg-d-dosed group F_0 males was statistically significantly lower than controls between SD 1 and SD 44, however, the terminal mean body weight was not statistically significantly different than control. The effect on body weight in 10 mg/kg-d dose-group males did not appear to be related to decreased feed consumption. In a two-generation reproduction and developmental study with the PFHxS congener, PFOS, with daily gavage dosing, F_0 male body weights were lower than controls at doses of 1.6 and 3.2 mg K^+PFOS/kg/d, becoming statistically significant by days 56 and 36 in these treatment groups, respectively [51]. Feed consumption and body-weight gain were also reduced with statistical significance by K^+PFOS treatment at doses ≥0.4 mg/kg-d. These data suggest that PFOS may be more effective in adversely affecting body weight in male SD rats than PFHxS at equivalent doses. This greater potency could be due to pharmacokinetic differences, pharmacodynamic differences, or both of these factors. Based on data presented by Luebker et al., the mean μM concentrations of PFOS in serum by dose were 91, 305, and 547 for the 0.4, 1.6, and 3.2 mg/kg-d dose F_0 male treatment groups, respectively. Mean liver PFOS concentrations in μM for the 0.4, 1.6,

and 3.2 mg/kg-d dose F_0 male treatment groups from Luebker et al. were 353, 647, and 2719, respectively. Based on data presented in this article, for the 10 mg/kg-d K^+PFHxS-treated F_0 males, the mean μM PFHxS concentrations in serum and liver were 594 and 1487, respectively. Considering that the K^+PFOS-treated F_0 males in the Luebker et al. study had been dosed approximately three weeks longer than those from the study reported herein, it is apparent that PFOS may be more potent than PFHxS. At doses of 1.0 mg/kg-d or higher, K^+PFHxS-treated F_0 male rats appeared to have reached serum steady state by SD 14 as their serum PFHxS concentrations were not statistically different between SD 14 and SD 42. In contrast, serum PFHxS concentrations in F_0 females were 12–23% of the F_0 males on SD 14. This gender difference in body burden may have contributed to the absence in body-weight effect in F_0 females as compared to males in each K^+PFHxS treatment group. In addition, differences in pharmacodynamic response cannot be ruled out.

The only effect of treatment with K^+PFHxS on organ weights observed were 20 and 56% increases in mean liver weight in F_0 male rats in the 3 and 10 mg/kg-d treatment groups, respectively, when compared to F_0 male control mean liver weight. Liver weight relative to either body weight or brain weight was also increased to a similar extent in the 3 and 10 mg/kg-d dose-group males as compared to controls. The incidence and severity of centrilobular hepatocellular hypertrophy observed correlated well with liver weight observations.

In the study reported herein, the mean serum and liver concentrations in F_0 males associated with no effect on liver weight at the 1 mg/kg-d dose were 89 μg/mL and 150 μg/g, respectively, after six weeks of K^+PFHxS treatment. At the effective K^+PFHxS dose of 3 mg/kg-d, mean serum and liver PFHxS concentrations in males were 129 μg/mL and 339 μg/g, respectively. It is interesting to note that, in data from a currently unpublished 3M study, daily gavage dosing of male and female SD rats with potassium PFOS at a dose of 1 mg/kg-d for 28 days resulted in increased liver-weight-to-body-weight ratios in the absence of a statistically significant effect on body weight. This effect was noted at mean serum and liver PFOS concentrations of 46 μg/mL and 107 μg/g for males, respectively, and 74 μg/mL and 126 μg/g for females, respectively. This observation would suggest that PFOS may be more potent in inducing hepatic hypertrophy than PFHxS.

The observed increase in mild to moderate thyroid follicular epithelial hypertrophy/hyperplasia in the 10 mg/kg-d treatment group males was consistent with the increase in centrilobular hepatocellular hypertrophy [50]. Thyroid hormones were not measured, but prior work with PFOS has demonstrated that competition for binding resulting in increased displacement of thyroid hormones as well as potential induction of increased metabolism of thyroid hormones by liver results in hypothyroxinemia without a strong compensatory increase in pituitary thyrotropin (thyroid-stimulating hormone or TSH) [52]. This is not the result of decreased pituitary release of TSH or response to hypothalamic thyrotropin releasing hormone. Increased hepatocellular hypertrophy in rats is often accompanied by increased thyroid follicular epithelial hypertrophy/hyperplasia as a consequence of induction of thyroid hormone metabolism [50,53]. A two-year dietary study conducted with K^+PFOS in Sprague Dawley rats did not produce an increase in thyroid follicular cell tumors in rats given K^+PFOS continuously throughout the study. However, male rats at the high dose level (20 ppm in diet) that were given potassium PFOS for the first year of the study and control diet thereafter did have an increase in thyroid follicular cell adenoma [54]. Whether the latter observation was a chance occurrence or represents a true effect remains to be determined.

K^+PFHxS treatment resulted in mild but statistically significant decreases in RBC count, hematocrit, and hemoglobin in male rats in the 3 and 10 mg/kg-d treatment groups. Histological examination

of bone marrow in the 10 mg/kg-d dose-group males did not reveal any abnormalities as compared to controls. Taking the hematology data from the study as a whole, this pattern would suggest slight to mild plasma volume expansion; although other potential etiologies cannot be ruled out with the available data. A similar pattern has observed with the PFHxS homolog, perfluorobutanesulfonate (PFBS) [55]. The PFHxS homolog, PFOS, has been demonstrated to be both a PPARα [56–59] and a PPARγ [56] agonist; although Takacs and Abbott did not observe activation of PPARγ with PFOS [60]. Plasma volume expansion is a known effect of thiazolidinediones mediated via PPARγ stimulation of the epithelial sodium channel (ENaCγ) of the collecting duct in kidneys [61]. Typically, weight gain is a consequence of fluid retention with thiazolidinediones, and this was not observed with PFHxS. Indeed, K$^+$PFHxS treatment caused decreased weight gain at the highest dose in males. In addition, no increases in sodium levels were noted. Dual activation of PPARγ and PPARα could mask a weight gain effect due to the increased β-oxidation associated with PPARα activation. Additional investigation would be required to develop a better understanding of the etiology of the reduced RBC count, hematocrit, and hemoglobin occurring in male rats given PFHxS.

The statistically significant increase in serum urea nitrogen (BUN) in 10 mg/kg-d dose-group parental males without a change in serum creatinine is suggestive of mild dehydration. However, this would be inconsistent with the observed small but significant decreases in RBC count, hematocrit, and hemoglobin, which are suggestive of volume expansion. The mean control value for BUN was consistent with the reported control values for the sex and strain for fasted rats [62]. BUN could also be increased as a result of increased protein catabolism [63]. With the information available from the study, it is difficult to establish the cause of the apparent increase in BUN.

The statistically significant effects observed on prothrombin time in male rats given K$^+$PFHxS were slight and did not follow a specific trend. These may represent normal variation in this parameter, as the values were within the range of normal (mean 13.6 ± 2.7 s) for 10–20-week-old Sprague Dawley rats as reported by Matsuzawa et al. [62].

The mild hypercalcemia observed in male rats at the 10 mg/kg-d dose is likely due to the increase in serum albumin also occurring at this dose. The ratio of serum albumin to calcium concentration is relatively constant across doses for males. Serum calcium should be corrected for serum albumin in cases where serum albumin is known to be altered [63]. In 10 mg/kg-d dose-group parental males, serum albumin increased over controls by about 5%, with serum calcium increasing by about 6%. Less likely potential etiologies of the mild increase in serum calcium would be an effect on parathyroid function or slight hyperthyroid state. The 37% increase in mean serum alkaline phosphatase in 10 mg/kg-d dose-group males compared to controls, while of unknown origin, would support either of these scenarios. However, increased serum albumin is the simplest and most supportable explanation.

The effect of K$^+$PFHxS treatment on decreasing serum total cholesterol in male rats was the most pronounced effect observed, occurring at all dose levels. Although not reaching statistical significance until the 10 mg/kg-d dose, mean serum triglycerides were also lower in all K$^+$PFHxS-treated male groups compared to the male control group. This observation is consistent with cholesterol reduction as a sensitive clinical endpoint for response to the PFHxS congener, PFOS [36,37]. The mechanism resulting in the reduction in serum total cholesterol with PFOS remains to be elucidated. In Wistar rats fed PFOS, decreased activities of hydroxymethylglutaryl CoA reductase and acyl-CoA:cholesterol acyl transferase and increased fatty acid oxidation leading to decreased lipoprotein particle production due reduced synthesis and esterification of cholesterol have been suggested [64]. Stimulation of PPARα is likely

involved in the case of PFOS [56,59,60]. These modes of action are currently being further investigated with PFHxS. Wolf et al. have demonstrated that PFHxS may actually be a more potent activator of mouse and human PPARα than either the four-carbon PFBS or eight-carbon PFOS in transiently transfected COS-1 cells [58].

The lack of effect of K$^+$PFHxS treatment on mating and fertility is consistent with prior observations with the congener, PFOS [51,65], and the perfluoroalkyl carboxylate, PFOA [66]. The dosing period did not allow for a complete cycle of spermatogenesis in the males.

In F_0 rats, the PFHxS concentrations in plasma and liver correlated well with the daily K$^+$PFHxS doses given. Prior to mating, the body burden (represented as plasma PFHxS concentration) in F_0 rats appeared to be sex-dependent: the plasma PFHxS concentrations in F_0 males were 4 to 8 times higher than that measured in F_0 females on SD 14.

Even though none of the F_1 rats received K$^+$PFHxS treatment directly, it was evident that neonates can receive PFHxS via in utero and lactational exposures. The same dose-dependent increases in serum and liver PFHxS concentrations were also observed in F_1 rats per daily K$^+$PFHxS doses given to the litter-matched F_0 female rats. The finding that serum and liver PFHxS concentrations in pups at PND 22 were higher than GD 21 indicated that exposure of F_1 progeny to PFHxS via the breast milk is as robust as that which occurs in utero across the placenta. Fetal serum PFHxS concentrations were slightly higher than maternal serum PFHxS concentrations on GD 21 in all treatment groups except for the highest treatment group. In both F_0 females and F_1 rats, liver PFHxS concentrations were lower than the respective serum PFHxS concentrations at all times.

While the ratio between F_0 male liver to serum PFHxS concentrations ranged from 1 to 3 on SD 42, the corresponding liver to serum PFHxS concentration ratios were approximately 0.5 or lower for all F_0 females on GD 21 and all F_1 pups on GD 21 and PND 22. Although there appeared to be a sex difference in serum and liver PFHxS concentrations in adult rats, there did not appear to be a sex difference between male and female F_1 rats throughout PND 22. The liver PFHxS concentrations remained similar between F_1 male and female rats in all treatment groups on PND 22. It is interesting that lack of a sex difference in PFOS serum and liver concentrations through PND 22 in rat pups from dams treated with K$^+$PFOS through gestation and lactation has also been reported [67], however, sex differences in serum PFOS concentrations became apparent after sexual maturation. Further study of the effect of sexual maturation on pharmacokinetic handling of PFHxS and PFOS would be of value.

The lack of significant postnatal effects in F_1 generation pups from K$^+$PFHxS-treated parents is quite different from the increased neonatal mortality and decreased postnatal growth observed for PFOS in Sprague Dawley rats and in mice (CD1 and SV129 PPARα wild-type and null) [51,65,68,69] and in CD1 and SV129 PPARα wild-type mice with PFOA [70–72]. The mean GD 21 maternal and fetal serum PFOS concentrations for the 10 mg/kg-d K$^+$PFHxS-treated dams (60 μg/mL and 44 μg/mL) approximated those of K$^+$PFOS-treated dams and their fetuses on GD 21 that were associated with significant reductions in postnatal pup survival (62 and 101 μg/mL, respectively) [65]. This observation suggests that K$^+$PFHxS treatment in rats is either not capable of producing neonatal mortality or requires higher body burdens in the fetal compartment.

PFHxS was first reported to be present at low ng/mL (ppb) concentrations in human serum in 2001 by Hansen et al. [11]. A number of biomonitoring studies of human populations subsequently have confirmed the widespread presence of PFHxS in samples from non-occupationally exposed populations [10,12–14,19–23]. These studies demonstrate that PFHxS can be found in umbilical cord blood serum collected in Canada [19], neonatal screening program blood spots from New York [10], and human milk samples from the

United States [20], China [21], and Sweden [22]. In addition, PFHxS has been found in plasma from children aged 5–6 from Bavaria [23], serum from children aged 2–12 from the United States [14], and serum from adolescents aged 12–19 from the United States [12,13] as well as adults [12,13,15–18].

The serum PFHxS concentrations in children from the United States are greater at the upper tails of the distribution than those of adults [14]. Olsen et al. [14] found that geometric mean serum PFHxS values among 598 children aged 2–12 from samples collected between 1995 and 1996 were not appreciably different than those of 645 American Red Cross blood donors taken between 2000 and 2001 [16] or 238 elderly blood donor serum samples from 1996 [15]. However, 67 of the 598 children (11%) had serum PFHxS concentrations above 30 ng/mL, as opposed to just one adult in each of the adult studies just cited. Similarly, Calafat et al., in reporting data from serum samples taken during 1999–2000 [12] and 2003–2004 [13] as part of the National Health and Nutrition Examination Survey (NHANES), demonstrated that, during both time periods, adolescents aged 12–19 years had the higher geometric mean serum PFHxS concentrations than the other three age groups (20–39 years, 40–59 years, and ≥60 years) and that the estimated 95th percentile serum PFHxS concentration of these adolescents was significantly greater than that of the three other age groups.

Exposure of children to PFHxS may begin very early in life. Monroy et al. [19] studied PFHxS serum concentrations in 101 pregnant women and their matched umbilical cord serum at birth. They found that umbilical cord blood serum at birth had a slightly greater mean PFHxS concentration than the respective mean for maternal serum at delivery (5.05 ± 12.92 ng/mL for umbilical cord blood serum versus 4.05 ± 12.30 ng/mL for mothers' serum); however, PFHxS was detectable in 46% of mothers' serum at birth versus 21% of umbilical cord blood serum samples. Lactational exposure to PFHxS has also been demonstrated in human populations. Human milk concentrations have ranged from a median of 0.011 ng/mL in 19 samples from China [21], a mean of 0.0145 ng/mL in 45 samples from Massachusetts [20], and a mean of 0.085 ng/mL in 12 samples from Sweden [22]. In the Swedish study, the respective mean maternal serum concentration was 4.7 ng/mL.

Reflecting on the larger proportion of children with serum PFHxS concentrations greater than 30 ng/mL as compared to adults, Olsen et al. [14] suggested that differences in the exposure patterns of children as well as potential exposure to surfaces in the home treated with materials that may contain or release PFHxS may account for the greater PFHxS serum concentrations in the tails of the distribution. Kubwabo et al. [27] and Strynar and Lindstrom [28] demonstrated the presence of PFHxS in house dust. In the Kubwabo et al. study, PFHxS concentrations in house dust were positively associated with the presence and extent of carpeting in homes and negatively associated with the age of the homes, which was negatively associated with the presence of carpet. Because children may spend more time in the home and in contact with treated surfaces, and boys may be somewhat more prone to these types of exposures (boys were more likely to have higher serum PFHxS concentrations than girls in the studies reported by Olsen et al. [14] and Hölzer et al. [23]), these data combined with the biomonitoring data provide support for the suggestion made by Olsen et al. to explain the differences in the distributions of adults and children at the upper end.

Sources other than house dust or treated surfaces in the home may include exposure via water, as suggested by Hölzer et al. [23] and Ericson et al. [29]. In particular, Hölzer et al. found that maximum children's plasma PFHxS concentrations were greater than those of adults in two German towns, one with potential exposure via drinking water. PFHxS has been found, less frequently, in samples from various wildlife [73–77], and Falandysz et al. [24] found an association of PFHxS serum concentrations with consumption of

fish in the Baltic. However, Ericson et al. [78] were not able to detect PFHxS in market food sources from the Catalan area of Spain.

Although a complete quantitative description of PFHxS exposure from various sources is not feasible at this time, it has become evident that PFHxS blood concentrations in the general public in the United States are declining since 2000–2002 when the principal manufacturer, 3M Company, discontinued production of PFHxS based on evidence of widespread distribution in non-occupationally-exposed populations [10,13,17]. In analyzing data from the National Health and Nutrition Examination Survey (NHANES), Calafat et al. [13] reported a 10% decline in PFHxS serum concentrations between 1999–2000 and 2003–2004. Using newborn screening blood spots for the same time period, Spliethoff et al. [10] reported a 23% decline. Similarly, Olsen et al. [17] showed a 30% decline in measured serum PFHxS concentration with American Red Cross blood donors between 2000–2001 and 2006. These declines appear to be consistent with the reported geometric mean serum PFHxS elimination half-life for 26 retired fluorochemical workers of approximately 7 years [7]. However, in Germany, PFHxS serum concentrations have been shown to be rising between 1977 and 2004 [26].

5. Conclusion

Treatment of mature adult Sprague Dawley rats with K^+PFHxS by daily oral gavage at doses up to 10 mg/kg-d for two weeks prior to mating, and for females, through gestation and lactation, and, for males, for a minimum of 42 days, did not produce major detrimental effects on mating, fertility, birth outcome, and development of offspring. The most sensitive effect, observed in parental males, was a decrease in total serum cholesterol, observed at all treatment levels.

Conflict of interest statement

John L. Butenhoff, Shu-Ching Chang, and David J. Ehresman are employees of 3M Company, a former manufacturer of K^+PFHxS and the company supporting the work reported on in the article. Raymond G. York does not have competing interests other than employment in contract research facilities (Charles River Preclinical Services) conducting parts of the work.

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